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ORIGINAL ARTICLE

Rebuild, restore, reinnervate: do human tissue engineered dermo-epidermal skin analogs attract host nerve fibers for innervation?

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Abstract
Purpose Tissue engineered skin substitutes are a promising tool to cover large skin defects, but little is known about reinnervation of transplants. In this experimental study, we analyzed the ingrowth of host peripheral nerve fibers into human tissue engineered dermo-epidermal skin substitutes in a rat model. Using varying cell types in the epidermal compartment, we wanted to assess the influence of epidermal cell types on reinnervation of the substitute.
Methods We isolated keratinocytes, melanocytes, fibroblasts, and eccrine sweat gland cells from human skin biopsies. After expansion, epidermal cells were seeded on human dermal fibroblast-containing collagen type I hydrogels as follows: (1) keratinocytes only, (2) keratinocytes with melanocytes, (3) sweat gland cells. These substitutes were transplanted into full-thickness skin wounds on the back of immuno-incompetent rats and were analyzed after 3 and 8 weeks. Histological sections were examined with regard to myelinated and unmyelinated nerve fiber ingrowth using markers such as PGP9.5, NF-200, and NF-145.
Results After 3 weeks, the skin substitutes of all three epidermal cell variants showed no neuronal ingrowth from the host into the transplant. After 8 weeks, we could detect an innervation of all three types of skin substitutes. However, the nerve fibers were restricted to the dermal compartment and we could not find any unmyelinated fibers in the epidermis. Furthermore, there was no distinct difference between the constructs resulting from the different cell types used to generate an epidermis.
Conclusion Our human tissue engineered dermo-epidermal skin substitutes demonstrate a host-derived innervation of the dermal compartment as early as 8 weeks after transplantation. Thus, our substitutes apparently have the capacity to attract nerve fibers from adjacent host tissues, which also grow into grafts and thereby potentially restore skin sensitivity.

Keywords Human skin analog · Nerve fibers · Innervation · Rat model

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Introduction

When full-thickness skin is lost, all intricate mechanisms perceiving and conducting what is known as normal skin sensibility are also completely lost. Whatever means of defect coverage is used (full-thickness skin, split-thickness skin, biosynthetic skin substitutes, cultured cells, and combinations thereof), initially there is no sensibility present. From a rather limited number of clinical articles we know that some sort of protective sensibility (sensory function) is restored over time, but sensory thresholds stay elevated and do not return to fully normal after skin grafting [1–6].

Although a variety of cultured skin substitutes have been produced over the last decades, some of them even for clinical applications, little is known about the chain of events leading to reinnervation and recovery of sensory function of the reconstructed skin. Our own laboratory has been working since more than 12 years on tissue engineering full-thickness analogs consisting of a stratified epidermis, a basement membrane, and a dermis [7–9]. Moreover, we even managed to insert human vascular endothelial cells into these constructs so as to obtain a prevascularization of the dermal compartment in vitro [10]. Lastly, we described that adding an appropriate number of melanocytes to these constructs restores an almost natural skin color [11].

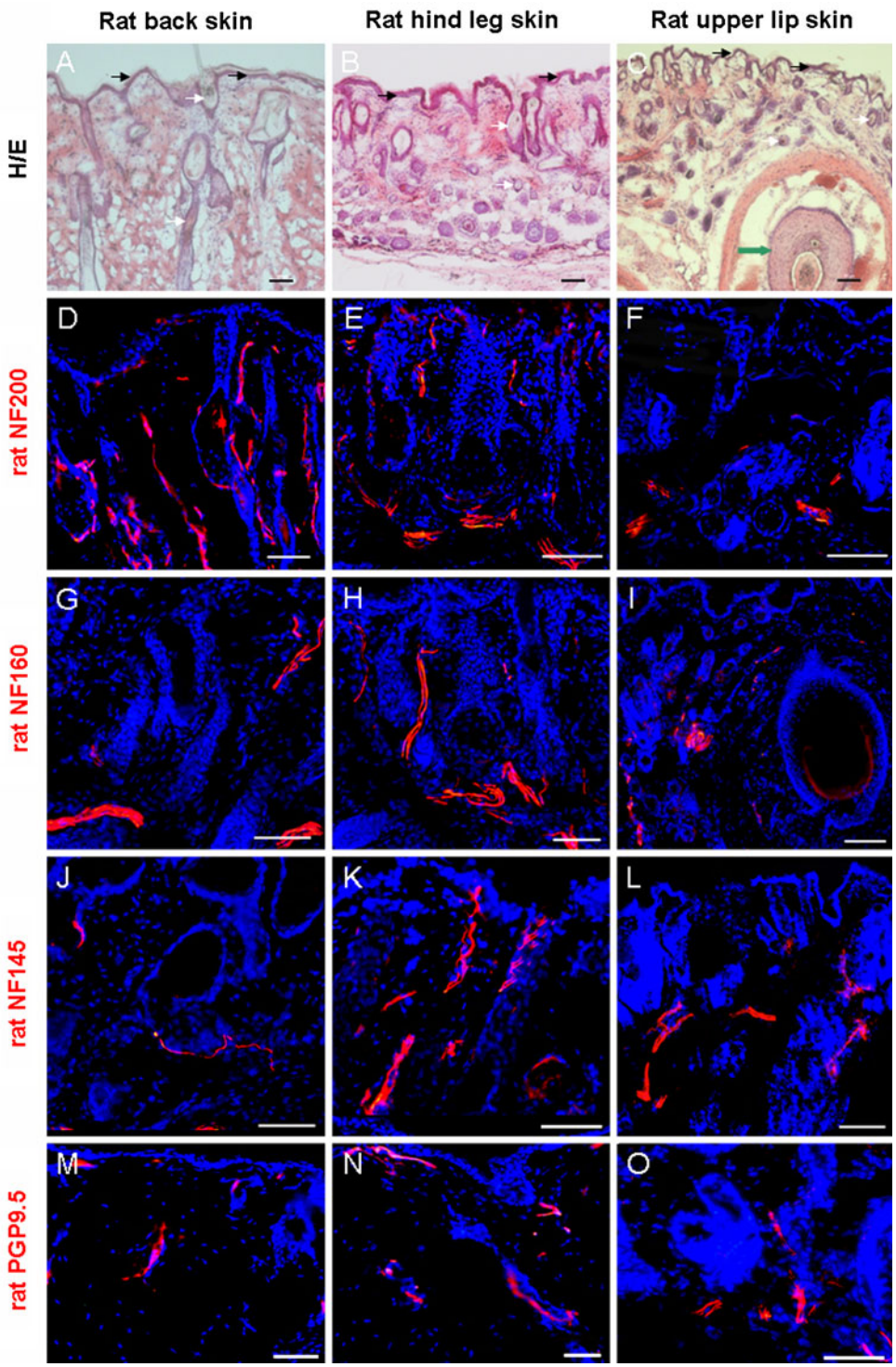

Fig. 1 Evaluation of different areas of normal rat skin (back, hind legs, upper lip). **a**, **b**, **c** Haematoxylin and Eosin staining of normal rat skin showing a thin epidermis (*black arrows*), abundant hair follicles (*white arrows*) in the dermis, and a whisker follicle (*green arrow*). **d–o** Nerve fiber staining of normal rat skin with NF200, NF160, NF145, and PGP9.5 (*red*). Cell nuclei are stained with Hoechst (*blue*). Expression of all neuronal markers can be observed in all rat skin specimens. *Scale bars* 100 μm

In this article, we present our preliminary data with regard to ingrowth of host nerve fibers into transplanted bioengineered human skin analogs.

Materials and methods

Human skin samples

The study was conducted according to the Declaration of Helsinki Principles and after permission by the Ethic Committee of the Canton Zurich. Informed consent was given by parents or patients. Human foreskins or skin samples from scalp or abdomen were obtained from patients ranging in age between 1 and 18 years. The skin samples were used for the isolation of human sweat gland cells, keratinocytes, melanocytes, and fibroblasts.

Isolation and culturing of primary cells

Keratinocytes and fibroblasts were isolated and cultured as described by Pontiggia et al. [7], sweat gland cells as described by Biedermann et al. [8], and melanocytes as specified in Böttcher-Haberzeth et al. [11].

Preparation of dermo-epidermal skin analogs

Skin analogs were prepared using a transwell system consisting of six well cell culture inserts with 3.0 μm pore-size membranes (BD Falcon, Switzerland) [7]. To build the dermal compartment, the membranes were covered with collagen type I hydrogels containing human dermal fibroblasts. Rat tail collagen type I (0.7 ml) (BD Biosciences, Switzerland) was mixed with 0.2 ml chilled neutralization buffer containing 0.15 M NaOH and 1×10^5 fibroblasts

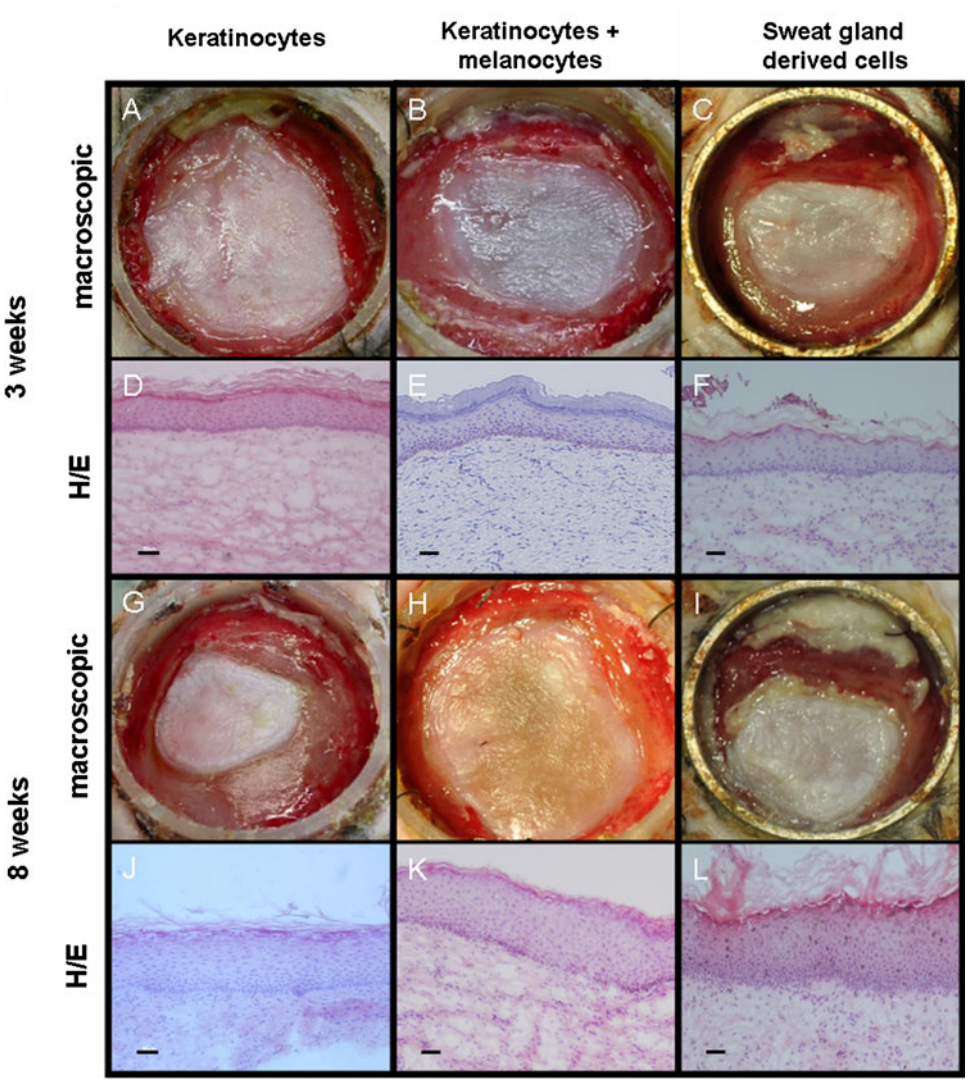

Fig. 2 Tissue engineered human skin analogs constructed with either keratinocytes, keratinocytes and melanocytes, or with sweat gland derived cells 6 and 8 weeks after transplantation. **a–c** Macroscopic view of the skin analogs 6 weeks after transplantation. **d–f** Haematoxylin and Eosin staining of the excised skin analogs 3 weeks after transplantation. **g–i** Macroscopic view of the skin analogs 8 weeks after transplantation. **j–l** Haematoxylin and Eosin staining of the excised skin analogs 8 weeks after transplantation. All constructs demonstrate both a macroscopic and microscopic appearance that, except for the absence of rete ridges and skin appendages, closely resembles normal human skin. *Scale bars* 50 μm

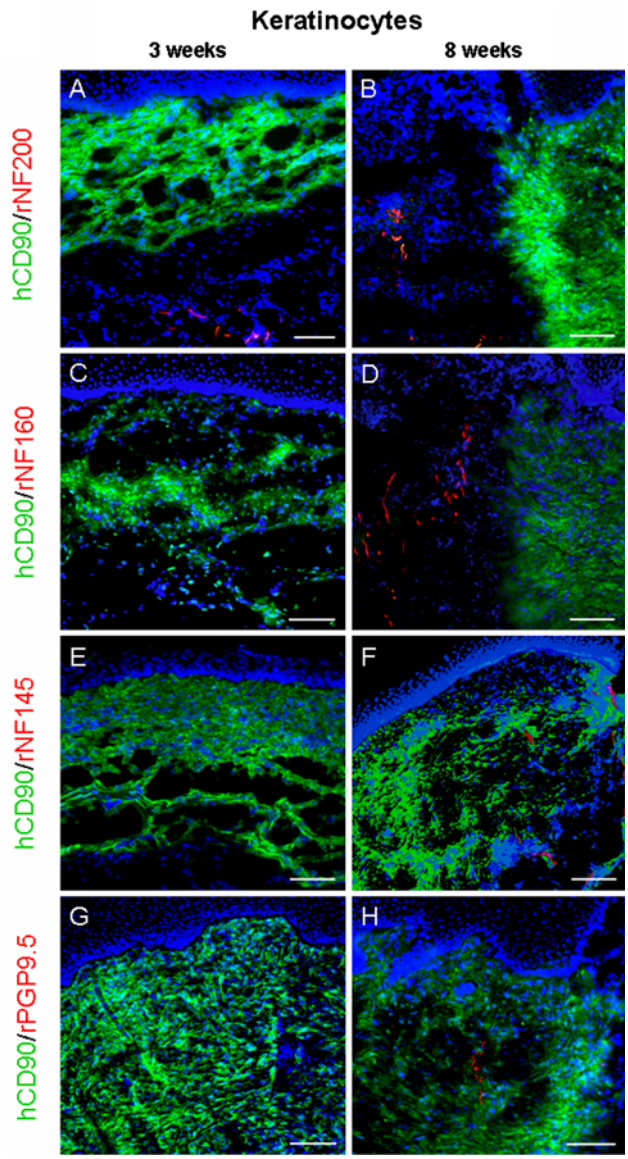

Fig. 3 Host innervation of human tissue engineered skin analogs constructed with keratinocytes. **a**, **c**, **e**, **g** Rat nerve fibers are stained with NF200, NF160, NF145, PGP9.5 (*red*) 3 weeks after transplantation. **b**, **d**, **f**, **h** Rat nerve fibers are stained with NF200, NF160, NF145, and PGP9.5 (*red*) 8 weeks after transplantation. Human dermal fibroblasts are stained with CD90 (*green*), cell nuclei are stained with Hoechst (*blue*). No innervation can be seen in the neodermis after 3 weeks, whereas NF145 and PGP9.5 stain positive in the constructs after 8 weeks. *Scale bars* 100 μm

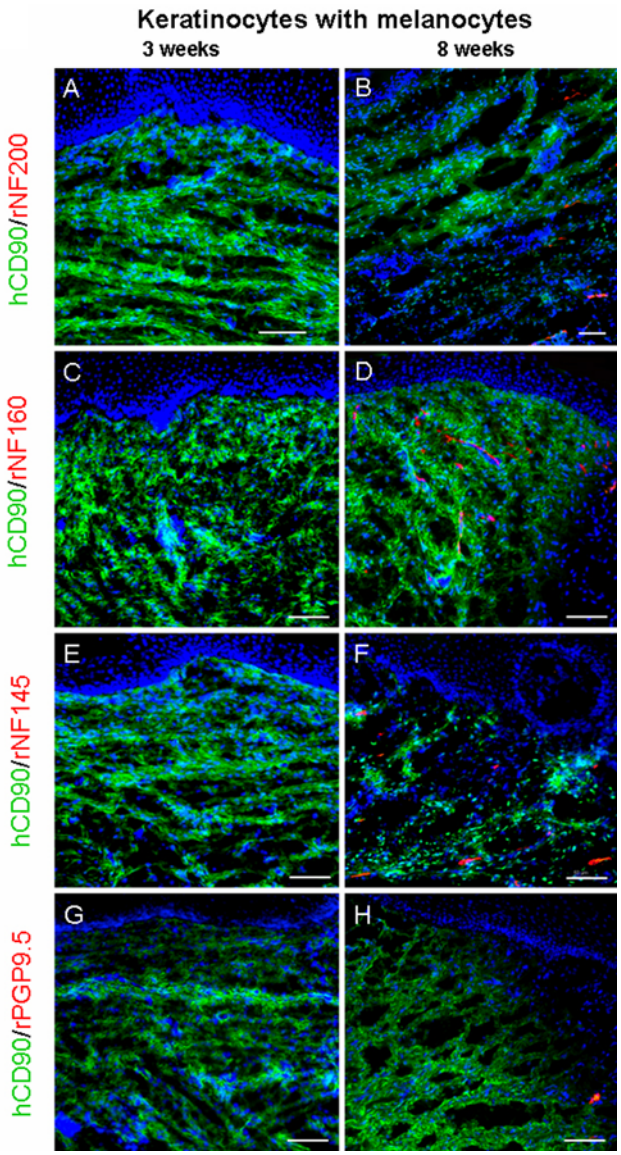

Fig. 4 Host innervation of human tissue engineered skin analogs constructed with keratinocytes and melanocytes. **a**, **c**, **e**, **g** Rat nerve fibers are stained with NF200, NF160, NF145, and PGP9.5 (*red*) 3 weeks after transplantation. **b**, **d**, **f**, **h** rat nerve fibers are stained with NF200, NF160, NF145, and PGP9.5 (*red*) 8 weeks after transplantation. Human dermal fibroblasts are stained with CD90 (*green*), cell nuclei are stained with Hoechst (*blue*). No innervation can be seen in the neodermis after 3 weeks, whereas all markers stain positive in the dermal compartment of the constructs after 8 weeks. *Scale bars* 100 μm

(passage 1). After polymerization (10 min at room temperature and 20 min at 37 °C), the dermal equivalents were cultured in DMEM/10 % FCS for 5 days. Subsequently, keratinocytes, or keratinocytes and melanocytes (ratio 5:1), or sweat gland cells were seeded onto each dermal equivalent at a density of 4×10^5 cells. The skin analogs were cultured in SFM (Invitrogen, Switzerland) for 1 week before transplantation. The medium was changed every second day.

Transplantation of cultured dermo-epidermal skin analogs

The surgical protocol was approved by the local committee for Experimental Animal Research (permission numbers 65/2009, 76/2011). Immuno-incompetent female nu/nu rats, 8–10 weeks old (Harlan Laboratories, Netherlands), were prepared and anesthetized as previously described

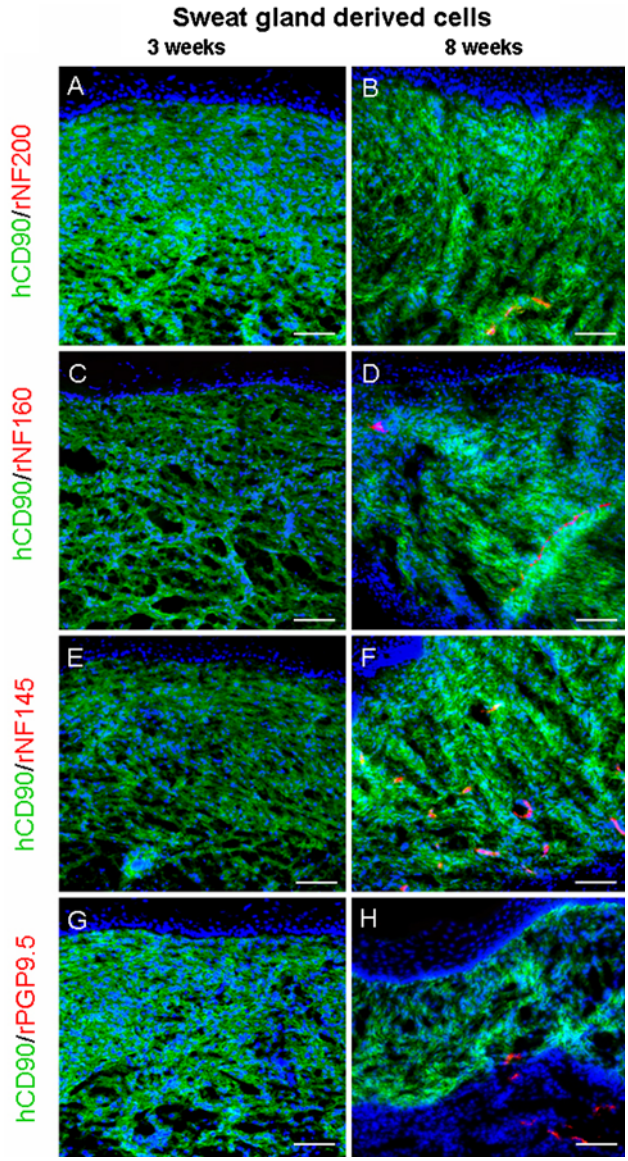

Fig. 5 Host innervation of human tissue engineered skin analogs constructed with sweat gland derived cells. **a**, **c**, **e**, **g** Rat nerve fibers are stained with NF200, NF160, NF145, PGP9.5 (*red*) 3 weeks after transplantation. **b**, **d**, **f**, **h** Rat nerve fibers are stained with NF200, NF160, NF145, and PGP9.5 (*red*) 8 weeks after transplantation. Human dermal fibroblasts are stained with CD90 (*green*), cell nuclei are stained with Hoechst (*blue*). No innervation can be seen in the neodermis after 3 weeks, whereas all markers stain positive in the dermal compartment of the constructs after 8 weeks. *Scale bars* 100 μm

[12, 13]. To protect the skin analogs and to prevent wound closure from surrounding rat skin, custom made surgical steel rings (diameter 2.6 cm) were sutured into full-thickness skin defects created on the backs of the rats, using non-absorbable polyester sutures (Ethibond®, Ethicon, USA). The transplants were then covered with a silicone foil (Silon-SES, BMS, USA), a polyurethane sponge (Ligasano, Ligamed, Austria), and a tape as wound dressing. Dressing changes and photographic documentations were performed once per week.

Analysis of the transplants

After 3 or 8 weeks, animals were killed by CO_2. Transplants and rat control skin were excised and embedded in OCT compound (Sakura Finetek/Digitana AG, Switzerland). Cryosections were stained with haematoxylin and eosin (Sigma, USA), and mounted within Eukitt® (Fluka, Switzerland) for histological analysis.

Double immunofluorescence stainings were performed to visualize myelinated and/or unmyelinated rat nerve fibers [ubiquitin carboxyl-terminal hydrolase L1 (PGP9.5, clone 13C4/J3C4, 1:50, Abcam, Germany), 200 kDa neurofilament (NF200, clone NF01, 1:50, Abcam, Germany), 160 kDa neurofilament (NF160, clone NN18, 1:50, Sigma-Aldrich, Germany), 145 kDa neurofilament (NF145, clone 3H11, 1:50, Chemicon, Germany)], rat vascular endothelial cells [CD31 (clone TDL-3A12, 1:50, BD Pharmingen, Switzerland)], and human fibroblasts [CD90 (clone AS02, 1:50, Dianova, Germany)]. Cryosections were fixed and permeabilized in acetone/methanol for 5 min at −20 °C, air dried, and washed 3× in phosphate-buffered saline (PBS, Invitrogen, Switzerland). Thereafter, sections were blocked in PBS containing 2 % BSA (Sigma, Switzerland) for 30 min. Incubation with the diluted first antibody (CD90 antibody) was performed in blocking buffer for 1 h at room temperature. Slides were washed three times for 5 min in PBS and blocked for additional 15 min. To visualize the primary antibody, FITC-conjugated polyclonal goat $F(ab')_2$ fragments directed to mouse immunoglobulins (Dako, Switzerland) were added to the sections. Slides were washed three times for 5 min in PBS and blocked for additional 15 min. For double immunofluorescence, NF and CD31 primary antibodies were prelabeled with Alexa 555-conjugated polyclonal goat $F(ab')_2$ fragments according to the instructions of the manufacturer (Zenon Mouse IgG Labeling Kit; Molecular Probes/Invitrogen, Switzerland) and added to the sections. Finally, the slides were incubated for 5 min in PBS containing 1 μg/ml Hoechst 33342 (Sigma, Switzerland), washed twice for 5 min in PBS, and mounted with Dako mounting solution (Dako, Switzerland).

Pictures of immunofluorescence stainings were taken with a DXM1200F digital camera connected to a Nikon Eclipse TE2000-U inverted microscope. The device is equipped with Hoechst 33342, FITC, and TRITC filter sets (Nikon AG, Switzerland; Software: Nikon ACT-1 vers. 2.70). Images were processed with Photoshop 7.0 (Adobe Systems Inc., Germany).

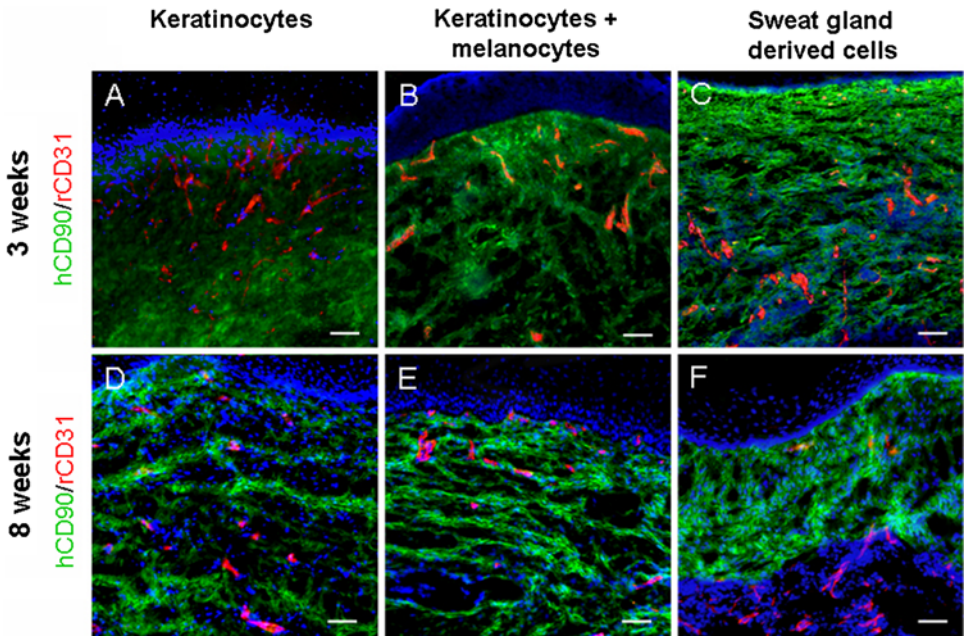

Fig. 6 Neovascularization of tissue engineered human skin analogs constructed with either keratinocytes, keratinocytes and melanocytes, or with sweat gland derived cells. **a–c** Skin analogs 3 weeks after transplantation. **d–f** Skin analogs 8 weeks after transplantation. Host endothelial cells are stained with CD31 (*red*), human dermal fibroblasts are stained with CD90 (*green*), cell nuclei are stained with Hoechst (*blue*). A rather dense capillary network throughout the entire neodermis can be seen after 3 and 8 weeks in all constructs. *Scale bars* 100 μm

Results

Expression of nerve fiber markers in normal rat skin

Native rat skin from back, hind leg, and upper lip was tested for nerve fiber marker expression (Fig. 1). All samples stained positive for NF200, NF160, and NF145 indicating the presence of myelinated nerve fibers. A marker detecting myelinated and unmyelinated nerve fibers (PGP9.5) also stained positive in all specimens.

Structure of human tissue engineered skin analogs tested

Three and eight weeks after transplantation, the human tissue engineered skin analogs constructed with the varying cell types in the epidermal compartment (keratinocytes, keratinocytes and melanocytes, sweat gland cells) were analyzed (Fig. 2). All constructs demonstrated complete take with a macroscopic appearance of normal human skin. Histologically, there was a correctly stratified epidermis (more than ten layers) and a compact and cellularized neodermis. In contrast to normal human skin neither hair follicles nor other skin appendages were present.

Host innervation of transplanted human tissue engineered skin analogs

After excision of the analogs 3 and 8 weeks post transplantation, the human neodermis (i.e., the site where nerve fibers physiologically reside) was evidenced by staining human fibroblasts (green, Figs. 3, 4, 5). After 3 weeks, no innervation in either of the three construct variations could be seen. After 8 weeks, host nerve fibers were detected in the dermal compartment of almost all specimens analyzed. In the analogs constructed with keratinocytes only, NF200 and NF160 only stained positive at the neodermal border (Fig. 3b, d), while NF145 and PGP9.5 stained positive within the neodermis. In all specimens, nerve fibers detected were restricted to the dermal compartment and no unmyelinated fibers could be identified in the epidermis. Furthermore, no difference was found regarding the different epidermal cell type variations.

Host vascularization of transplanted human tissue engineered skin analogs

Three weeks after transplantation, CD31 staining showed a capillary network throughout the dermal compartment of the human tissue engineered skin analogs (again delineated with human fibroblast staining) (Fig. 6). After 8 weeks, the

rat blood vessels were present in about the same way as 3 weeks post transplantation.

Discussion

Our data provide evidence that human cell-derived tissue engineered skin substitutes, transplanted onto immunoincompetent rats, exhibit host-sponsored sensory innervation. We have used classical markers, including NF200, NF160, NF145, and PGP9.5 [4–6, 14] to identify peripheral nerve fibers. With these markers, we were able to demonstrate that innervation occurs partially at 6 weeks post transplantation (data not shown) and is clearly identifiable in all specimens analyzed 8 weeks post transplantation. However, we only observed nerve fibers in the dermal compartment and not (yet) in the epidermis, where unmyelinated free nerve endings are physiologically present [15–17]. We hypothesize that epidermal innervation would occur only later and are currently testing this hypothesis using the same model.

Interestingly, the dynamics of nerve fiber ingrowth from underlying host tissue into grafts was independent of whether constructs containing keratinocytes alone, keratinocytes plus melanocytes, or sweat gland cells were used to generate an epidermis. Theoretically, the following explanations might account for the phenomenon described: keratinocytes, melanocytes, and sweat gland cells all do not play a role, or, with regard to graft innervation, they all play the same role in attracting neuronal ingrowth. Alternatively, rat nerve fibers do not apparently have preferential epidermal partner cells when growing into and through the dermal compartment towards the epidermis.

Finally, there is an interesting difference between the timelines of graft vascularization and graft innervation. An extensive rat capillary network can be identified as early as 3 weeks post transplantation in the dermal compartment, whereas a marked innervation of the dermal compartment of our skin analogs is present only at 8 weeks post transplantation. It is reasonable to assume that establishing of an appropriate vascular supply within the transplanted skin has the highest priority with regard to immediate graft take and long-term graft survival, and that it therefore occurs earlier. Yet, it is known that blood vessels and nerve fibers do crosstalk by releasing signals (e.g., VEGF, NGF), leading them to align themselves in close proximity of each other to form the so called neurovascular bundles. In light of this fact one could have imagined that vessels and axons would grow into grafts with a similar speed after transplantation, but this was apparently not the case in our model [18, 19].

Of note, the allogenic setting used in the presented study does not allow to draw any meaningful conclusions with regard to functional questions, in particular issues concerning the wide variety of quantitative (threshold determination) and qualitative (touch, pain, temperature, vibration) skin sensibility. However, we interpret the fact that bioengineered human skin is innervated by host nerve fibers as potentially favorable. We assume that in an analogous, but autologous setting, these innervation processes would take place in at least the same way as shown here, if not better.

In summary, we demonstrate for the first time, that tissue engineered dermo-epidermal substitutes of human origin exhibit a host-derived sensible innervation 8 weeks after transplantation. These skin analogs apparently have either the capacity to actively attract nerve fibers, or alternatively, the ability to (passively) allow nerve fiber ingrowth from the adjacent host tissue. It appears likely that the same favorable dynamics take place when autologous bioengineered skin analogs are transplanted onto patients.

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Conflict of interest The authors declare that they have no conflict of interest.

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